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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>4</sup> :</b> <b>C12Q 1/68, C07H 15/12</b> <b>G01N 33/48</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 90/01069</b> <b>(43) International Publication Date:</b> 8 February 1990 (08.02.90)
<b>(21) International Application Number:</b> PCT/US89/03125 <b>(22) International Filing Date:</b> 19 July 1989 (19.07.89)  <b>(30) Priority data:</b> 221,750      20 July 1988 (20.07.88)      US  <b>(71) Applicant:</b> SEGEV DIAGNOSTICS, INC. [US/US]; Greenfeld, 1115 52nd Street, Brooklyn, NY 11219 (US).  <b>(72) Inventor:</b> SEGEV, David ; Moshav Bnei Mrem 40, 79 840 (IL).  <b>(74) Agents:</b> LIEBERSTEIN, Stanley, H. et al.; Ostrolenk, Fa- ber, Gerb & Soffen, 1180 Avenue of the Americas, New York, NY 10036 (US).		<b>(81) Designated States:</b> AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK, FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> PROCESS FOR AMPLIFYING AND DETECTING NUCLEIC ACID SEQUENCES  <b>(57) Abstract</b> <p>This invention relates to a process for amplifying and detecting any desired specific nucleic acid sequence that exists in a nucleic acid or mixture thereof. The process comprises treating single strand RNA or separated complementary strands of DNA target with a molar excess of oligonucleotide complement pairs in which these oligonucleotide complement pairs have sequences complementary to the target, under hybridizing conditions. In one embodiment, the oligonucleotide complement pairs may have a gap of one or more bases which may be repaired (filled) by enzymes. The oligonucleotide complement pairs are joined together, forming joined, oligonucleotide product. The target/joint product hybrid nucleic acids are then denatured to single strands again, at which point both the target and the joined products can form hybrids with new oligonucleotide complement pairs. The steps of the reaction may be carried out stepwise or simultaneously and can be repeated as often as desired.</p>		

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PROCESS FOR AMPLIFYING AND  
DETECTING NUCLEIC ACID SEQUENCES

FIELD OF THE INVENTION

5       The present invention is related to a process  
for amplifying and detecting existing nucleic acid  
sequences if they are present in a test sample. More  
specifically, it is related to a process for producing  
any particular nucleic acid sequence from a given  
sequence of DNA or RNA in amounts which are large, when  
10       compared to the amount initially present. The DNA or  
RNA may be single or double-stranded, and may be a  
relatively pure species or a component of a mixture of  
nucleic acids. The process of the invention utilizes a  
repetitive reaction to accomplish the amplification of  
15       the desired nucleic acid sequence.

BACKGROUND OF THE INVENTION

For diagnostic applications in particular,  
the target nucleic acid sequence may be only a minute  
portion of the total pool of DNA or RNA in a sample to  
20       be screened, so that it may be difficult to detect the  
presence of the target nucleic acid sequence using  
nonisotopically labeled or end-labeled oligonucleotide  
probes. Thus, diagnostic tests employing DNA probes to  
detect rare species of nucleic acids are often not  
25       sensitive enough to be practical for use outside of the  
research laboratory.

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One attempt to overcome the sensitivity problem is the polymerase chain reaction (PCR) method, described in U.S. Patent Nos. 4,683,195 and 4,683,202 ("the '195 and '202 patents"). This method proceeds  
5 basically, as follows:

- a) treating a sample suspected of containing the target nucleic acid sequence of interest with one oligonucleotide primer for each strand of the target nucleic acid sequence, under  
10 hybridizing conditions and in the presence of a polymerase, e.g., the Klenow fragments of Escherichia coli DNA polymerase-I, such that an extension product of each primer is synthesized if the target nucleic acid  
- 15 sequence is present;
- b) placing the sample after step (a) under denaturing conditions to separate any primer extension products that were synthesized from the templates on which they were synthesized  
20 to produce single-stranded molecules;
- c) treating the single-stranded molecules generated from step (b) with the primers of step (a) under the conditions of step (a), such that the new primer extension products  
25 are synthesized using both the original target sequences and the primer extension products produced in step (a) as templates, thus resulting in the amplification of the target nucleic acid sequence.

30 Steps (a)-(c) may be conducted sequentially or simultaneously. In addition, steps (b) and (c) may be repeated until the desired level of sequence

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amplification is obtained. As discussed in U.S. Patent Nos. 4,683,195 and 4,683,202, the product of step (c) may be detected using probes.

5 The PCR method has a disadvantage in that it fails to completely overcome the sensitivity problem. The PCR method uses all four nucleotide bases to extend the primer fragments. Therefore, extension products may be created from other, non-target nucleic acid templates that may be present in the sample such as  
10 nicked, double-stranded DNA. The use of the PCR method results in considerable background of amplified DNA other than the target sequence(s).

As will be discussed in detail later, the present invention uses at least two oligonucleotides  
15 for each strand of target nucleic acid sequence and uses fewer than all four bases, thus reducing the problem of nonspecific, background amplification for a number of reasons. For example, when labeled nucleotides are used, the gap will be filled with  
20 labeled nucleotides if the nucleic acid target sequence exists in the sample and the irrelevant sequences will not be copied or labeled.

The polymerase chain reaction method also requires heat stable enzymes for the process to be  
25 automated, while the process of the present invention can be performed using heat-labile enzymes or without any enzymes, depending upon the particular embodiment. In addition, the detection of amplified nucleic acids produced in the PCR method often requires the use of  
30 gels or a capturing system, which are laborious detection methods. In contrast, the detection of the amplified sequences in the present invention is



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5 In one embodiment of the present invention,  
the amplification is accomplished by using two or more  
oligonucleotide complement pairs wherein at least one  
strand of these oligonucleotide pairs has a nucleotide  
sequence complementary to at least a portion of the  
target sequence. The oligonucleotides are selected so  
there is a gap of at least one base when the  
complementary strands of the "oligonucleotide  
10 complement pairs" and the target nucleotide are  
hybridized to one another. The gap between the  
oligonucleotide complement sequences is filled by a  
mixture of polymerase and ligase producing an  
oligonucleotide repair product. The resulting mixture  
of hybridized molecules is then placed under denaturing  
15 conditions.

After the strands separate during  
denaturation, the ligated oligonucleotide product can  
hybridize to its complementary strands from other  
oligonucleotide complement pairs, and then the gap is  
20 filled again. The process is repeated as often as is  
necessary to produce the desired amount of  
oligonucleotide repair product. In one embodiment of  
the present invention, the enzymes are immobilized on a  
polymeric support.

25 The present method is especially useful, for  
amplifying sequences indicative of a genetic disorder  
and rare species of nucleic acid present in a mixture  
of nucleic acids, and further permits the effective  
detection of such nucleic acid sequences. The present  
30 invention provides a process for amplifying at least  
one specific nucleic acid sequence in a sample of a  
nucleic acid or a mixture of nucleic acids. Each



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For any continuous base sequence A-Q-B or A'-Q'-B', Q or Q' can be composed of only one set of base pairs for any specific sequence, i.e., A-T, A-U, G-C, or derivatives of these bases. A-Q-B and A'-Q'-B' and are now joined, oligonucleotide products and can now serve as "target" sequences for other oligonucleotide complement pairs. When the joined, oligonucleotide product is formed by this gap-filling, ligated process it is termed an "oligonucleotide repair product."

- b) Treating the sample under denaturing conditions to separate the oligonucleotide repair products from their targets, if the nucleic acid target sequence(s) is(are) present.
- c) Treating the sample as in step (a) with oligonucleotide complement pairs A,A' and B,B' under hybridizing and gap-filling conditions such that an oligonucleotide repair product is obtained using each of the single strands produced in step (b), resulting in the amplification of the specific nucleic acid target sequence(s) if present.

The steps may be conducted sequentially or simultaneously. In addition, steps (b) and (c) may be repeated until the desired level of sequence amplification is obtained.

In another embodiment of the present invention, photosensitive molecules, x and y, are attached to each strand of the oligonucleotide

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complement pairs at the ends of the molecules which are to be joined together. X and y are compounds capable of forming carbon-carbon double bonds and undergoing [2 + 2] photocyclodimerization, thereby linking the oligonucleotide products when photoactivated and forming a joined, oligonucleotide product termed "an oligonucleotide photocyclodimerized product." In this embodiment, a gap between contiguous nucleotide strands is unnecessary. A gap of one or two bases is permissible.

This invention is also related to methods for the detection of the amplified specific nucleic acid sequence and diagnostic kits applicable thereto.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 illustrates a 48 base pair length sequence of HIV from GAG region desired to be amplified. The base pairs which fill the gap is depicted above and below the 48 base pair.

Figure 2 shows a 12% polyacrylamide-7M urea gel, where each lane consists of a cycle that includes: boiling the mixture of human immunodeficiency virus (HIV) plasmid target with labeled oligonucleotides, attached to photoreactive compounds, quick cooling the mixture, to 37°C and irradiating the mixture for five minutes.

Fig. 3 depicts an apparatus for carrying out one embodiment of the method of the present invention wherein the enzymes used are immobilized on a polymeric support.





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to contain the target nucleic acid sequence of interest under hybridizing conditions. The oligonucleotide complement pairs are selected so that there is a gap in the nucleotide sequence of at least one base between the two complements when the two complements are hybridized with the nucleic acid target sequence. The gap is then filled and the two oligonucleotides are ligated together, producing an oligonucleotide repair product. The process of gap-filling plus ligation is analogous to the repair of mismatched bases, and other errors that occur during DNA replication, repair of UV damage, and other processes in vivo. The nucleic acid target sequence and the oligonucleotide repair product sequence may then be separated and the process repeated over and over again until the desired level of amplification has been achieved. To avoid a problem of background synthesis occurring during the gap filling step, the two or more oligonucleotide complement pairs are selected so that the gaps between them will require less than all four bases to fill in the gap, preferably one set of complementary bases, namely A-T, A-U, or G-C. Without all four bases, random synthesis would not be initiated by nucleic acid sequences that may have nicks or are in the process of replication or transcription.

#### 1. Nucleic Acid Target Sequences

The process of the present invention can produce exponential quantities at least one specific nucleic acid sequence relative to the number of reaction steps involved, provided that (a) at least part of the nucleic acid target sequence is known in

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sufficient detail that oligonucleotide pairs can be synthesized which can hybridize to it, or (b) the target sequence can be isolated in large enough quantities to produce enough oligonucleotide complement pairs for use in the process. Any source of nucleic acid can be utilized as the source of the target nucleic acid sequence, in purified or nonpurified form. For example, the process may employ either single-stranded or double-stranded DNA or RNA. In addition, a DNA-RNA hybrid which contains one strand of each may be utilized. A mixture of any these nucleic acids may be employed. The specific nucleic acid sequence to be amplified may be only a fraction of a larger molecule. It may be a minor fraction of a complex mixture, such as a portion of the HIV (human immunodeficiency virus) gene integrated in the genomic DNA of an infected person, or bacterial nucleic acid present in very low quantities in a particular biological sample.

To determine the sequence of the target sequence(s), or as a sample to be tested, the nucleic acid or acids of interest may be obtained from any source, for example, DNA or RNA, isolated from bacteria, viruses, yeast and higher organisms such as plants or animals, from plasmids such as PBR 322 and M13, from cloned DNA or RNA by a variety of techniques known to those skilled in the art. DNA may also be extracted from cells grown in tissue culture by techniques such as those described by Maniatis et al., Molecular Cloning, a Laboratory Manual (New York: Cold Spring Harbor Laboratory, 1982), pp.280-281.





























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molecules and/or molecules having a particular length or sequence. For instance, the reaction mix may be passed through a Sephadex column to separate the labeled NTPs and the joined oligonucleotide products. Since individual nucleotides are much smaller than the length of the two or more oligonucleotide complement pairs, the detection of amplified material should be fairly simple based on size alone.

Another technique to detect amplified sequence would require the construction or isolation of probes that share complementary sequences with enough of each and every oligonucleotide complement pair to bind and hold the joined product preferentially. Such probes can be immobilized on any suitable substrate. They may also be labeled differentially if desired.

The present invention may be used for in vitro diagnostics. The process of amplifying nucleic acid sequences enables the detection of specific nucleic acid sequences associated with infectious disease, genetic disorders or cellular disorders such as cancer. Amplification is particularly useful when the amount of nucleic acid target available for diagnosis is in minute quantities, as, for example, in the prenatal diagnosis of sickle cell anemia, which requires obtaining DNA from fetal cells. Furthermore, it is within the ability of those skilled in the art, that the length and sequences of the oligonucleotide complements can be varied to detect deletions and/or mutations in genomic DNA from any organisms. These small changes are important in the diagnosis of such conditions as cystic fibrosis,  $\alpha$ -thalassemia,  $\beta$ -thalassemia and the like.



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radioactive ones. As described in EP 63,879 to Ward, biotin-containing DNA probes are detected by chromogenic enzymes linked to avidin or biotin-specific antibodies. This type of detection is convenient, but relatively insensitive. The combination of DNA amplification by the present method and the use of Bio-dUTP to label the bases that filled the gap could provide the convenience and sensitivity required to prepare useful diagnostic kits and to overcome the difficulties associated with both Falkow and Ward procedures when these techniques are applied in a routine clinical setting.

The use of the Falkow and Ward methods, the synthesis of oligonucleotides, the calculation of the number of sequences amplified per cycle, and other matters that pertain generally to amplification of nucleic acid sequences are described in the '195 and '202 applications and these applications are incorporated herein by reference.

The invention will now be illustrated by examples. The examples are not intended to limit the scope of the present invention. In conjunction with the general and detailed description above, the examples provide further understanding of the present invention and outlines some aspects of the preferred embodiments of the invention.

#### EXAMPLE 1

The desired sequence to be amplified using the gap-filling/ligating embodiment of joining the oligonucleotides is a 48 base pair sequence that coded for HIV at GAG region 2106-2153. This sequence is







































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After 3 hours of cycling the solution through cell 2 containing the immobilized enzymes, the solution is loaded on a Sephadex column G-40/50 (1 x 10 cm) and eluted with doubly distilled water. Fractions of 1 ml are collected and monitored by a Geiger counter.

Two distinct bands are detected, the first is eluted in a pool of 4-7 ml containing labeled amplified joined oligonucleotide products by gap filling/ligation process. This pool has about 115000 cpm whereas the second pool that eluted in 14-17 ml has 80,000 cpm.



























